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Hemodialysis and red cell cation transport in uremia: Role of membrane free fatty acids

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Hemodialysis and red cell cation transport in uremia: Role of membrane free fatty acids. Active and facilitated cation transport in erythrocytes of uremic patients may be improved acutely by hemodialysis, although the mechanisms remain unknown. As nonesterified fatty acids (NEFA) can affect Na^+ pump activity in vitro, changes in plasma and red cell membrane NEFA content following a single hemodialysis procedure were examined and compared with acute changes in erythrocyte cation flux rates in 34 hemodialysis patients. In nonsodium-loaded cells, small changes in Na^+ pump flux with dialysis did correlate with changes in intracellular Na^+ content ($r = 0.59$; $N = 17$; $P < 0.01$). On average, neither maximal Na^+ pump activity nor Na^+/Li^+ countertransport flux improved with dialysis, but $Na^+/K^+/Cl^-$ cotransport rates rose 25% post-dialysis ($P < 0.02$). Plasma NEFA levels rose 87% following hemodialysis but erythrocyte membrane NEFA content declined by 23% ($P < 0.001$). Importantly, 24 of the 34 subjects studied had a decrease in erythrocyte membrane NEFA content of greater than 10%, and in these patients, the fall in membrane NEFA correlated with an increase in ouabain-sensitive Na^+ efflux ($r = 0.564$; $P < 0.01$). The effects of hemodialysis on both erythrocyte NEFA content and Na^+ pump flux could be reproduced by incubating pre-dialysis cells in fatty acid-free albumin. We conclude that acute changes in membrane NEFA may modulate active cation transport in uremic erythrocytes.

Severe chronic renal insufficiency (CRF) and the uremic syndrome are characterized by a number of biochemical and physiologic abnormalities, including defects in transmembrane cation transport [1]. Although most investigators have examined active and facilitated cation transport pathways in the erythrocyte [2–9], observations in several other tissues, including leukocytes [10], skeletal muscle [11, 12], and adipose tissue [12] support the hypothesis that a generalized alteration in cation transport pathways exists in uremia. The activity of the Na^+ pump has been the most intensively studied ion transport system. Controversy remains as to whether a majority of uremic patients exhibit decreased Na^+ pump activity and/or number, but there appears to be a consensus that at least a subset of patients have reduced ouabain-sensitive cation flux rates, a defect that may be ameliorated acutely by hemodialysis [2, 4]. Several recent reports have also described a decrease in furosemide-sensitive $Na^+/K^+/Cl^-$ cotransport in uremic erythrocytes [7, 13], a defect we have also found in adipocytes and skeletal muscle of uremic rats [12].

Despite the considerable evidence for abnormal cation transport in uremic patients and in experimental animals with CRF, the mechanism for these changes remains unknown. Plasma factors, including uremic toxins and possibly endogenous Na^+ pump inhibitors, have been implicated since the early experiments of Cole, Balfe and Welt [2] who reported that uremic serum could transfer the defect in Na^+ pump activity to normal erythrocytes, abnormalities that have been confirmed by some [4], but not all, investigators [13]. There have also been many reports of factors with endogenous digitalis-like activity in the plasma of patients with uremia [14–16].

Recently, several laboratories [17, 18, 19], including our own [20, 21], have identified polar lipids in plasma, predominantly nonesterified fatty acids (NEFA) and certain lysophospholipids, as potential endogenous modulators of the Na^+ pump. We found that NEFA and other polar lipids could reversibly inhibit NaK-ATPase activity in vitro and inhibit ouabain binding to the cardiac glycoside binding site on NaK-ATPase. However, it has remained unclear whether polar lipid compounds in plasma could ever affect Na^+ pump activity or other ion transporters in a physiologically important manner in vivo. Since abnormalities both of lipid metabolism [22] and of plasma protein binding of NEFA occur in uremia [23], we wished to determine whether the changes in plasma NEFA levels that occur acutely with hemodialysis are paralleled by changes in red cell membrane NEFA content, and whether altering membrane NEFA levels could be correlated with changes in both active and facilitated transmembrane cation transport.

Methods

Preparation of red cell membranes

Blood was drawn into chilled heparinized tubes and kept on ice until processed; this was always less than 45 minutes. Whole blood was centrifuged, the buffy coat was removed, and the plasma was immediately extracted with organic solvents. Four ml of packed RBCs were resuspended in a preservation solution containing 140 mM KCl, 10 mM NaCl, 25 mM KPO_4, 10 mM Tris-Mops (pH 7.4 at 4°C) and 1 mM $MgCl_2$ (hematocrit 50%) and kept at 4°C until used for cation flux and [^{3}H]ouabain binding experiments. Another aliquot of cells was immediately washed four times in a choline wash solution (CWS: 149 mM choline Cl, 1 mM $MgCl_2$, 0.1 M Tris-Mops, pH 7.4, 4°C) and

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resuspended in a 1:50 dilution of 0.02% Acationox (American Scientific Products, Boston, Massachusetts, USA) to measure hematocrit and intracellular electrolytes. To determine the membrane NEFA content, 2 ml of RBCs were resuspended in an isotonic Tris buffer (172 mM Tris-Cl, pH 7.6 at 4°C, 310 mOsm) and washed twice to remove plasma. Five ml aliquots of a 50% suspension of washed RBCs were transferred to 50 ml polycarbonate tubes containing 40 ml of a 11.1 mM Tris hypotonic buffer (20 mOsm, pH 7.6 at 4°C). The tubes were vortexed vigorously, centrifuged at 20,000 g for 30 minutes at 4°C, and the supernatant was discarded. RBC membranes were resuspended in 5 ml of a sucrose buffer (0.25 M sucrose, 1 mM EDTA, 20 mM Tris-Cl, pH 7.4 at 4°C). A 100 μl aliquot was added to 100 μl of 0.05 M NaOH in 1% (vol/vol) sodium dodecyl sulfate, vortexed, and frozen at −20°C for later protein determination. Two 1 ml aliquots were immediately prepared for determination of red cell membrane NEFA; the remainder was stored at −20°C.

Determination of plasma and red cell membrane NEFA content

One ml aliquots of RBC ghost membranes in sucrose buffer were heated to 70°C for 15 minutes in glass centrifuge tubes under a constant stream of N_2 in order to inactivate phospholipases. This step was always performed immediately following phlebotomy and preparation of erythrocyte ghosts, with care taken to keep all buffers cold. Twenty-five nmoles of an internal standard ($C_{17:0}$; heptadecanoic acid, Sigma Chemical Co., St. Louis, Missouri, USA) were added, the tubes were vortexed and then 1 ml methanol was added and mixed by vortexing for 15 seconds. Three ml of chloroform/BHT (butylated hydroxytoluene, 50 mg/liter) solution were added, vortexed for one minute, centrifuged at 2,000 g for five minutes, and the organic layer was transferred to a second conical glass centrifuge tube. The aqueous layer was washed with a 1:3 mixture of methanol/chloroform, and the organic layers from the first and second wash were combined and dried under N_2. The extracted red cell membrane lipid mixture was redissolved in 1 ml of chloroform, and 200 μl aliquots were placed in 5 ml glass tubes to which an additional 300 μl of chloroform were added. Triplicate 500 μl aliquots were added to preparative low pressure aminopropyl-bonded phase columns (Bond-Elut, Analytichem International, Harbor City, California), to separate NEFA from other polar and neutral lipids according to the technique of Kaluzny et al [24]. The columns were eluted with 4 ml of a 2:1 mixture of chloroform:2-propanol to remove neutral lipids, and then 4 ml of 2% acetic acid in diethyl ether to remove NEFA. This second eluate was dried under N_2 and redissolved in 1 ml of chloroform/BHT and stored at −20°C until derivatization. This technique for separating complex lipid mixtures yielded recoveries of radiolabelled lipids of >95%, in agreement with the report of Kaluzny et al [24] and Kelly et al [25].

Plasma samples were kept on ice until lipids were extracted. This was done within 45 minutes of venipuncture. Two hundred and fifty μl aliquots of plasma were added to 250 μl of water containing 20 nmoles of hepatadecanoic acid. This was extracted twice in methanol/chloroform (1:3, vol/vol) containing 50 mg/ml BHT. The combined organic phases were dried under N_2, reconstituted in 1 ml chloroform, and applied to Bond Elut columns to separate NEFA as described above [25]. Following the separation of NEFA from other plasma lipids, they were stored in chloroform/BHT at −20°C until assayed.

NEFA from erythrocyte ghost membranes and plasma were quantitated using a modification [25] of the technique of Tsuchiya et al [26, 27]. Frozen lipid samples were dried under N_2, reconstituted in 1 ml of acetone, and derivatized with the fluorescent reagent, 4-bromomethyl, 7-acetoxy coumarin (Br-Mac), as described previously [25]. Derivatized NEFA were separated by reverse phase HPLC, with post-column alkaline hydrolysis of Br-Mac, while Br-Mac fluorescence was monitored at 365 nm excitation and 460 nm emission. The coefficient of variation was less than 5%, with at least 10 pmol of each NEFA injected for plasma samples, and less than 9% for red cell ghost membrane samples; the limit of detection was approximately 50 fmoles. All samples were run in duplicate or triplicate. Red cell ghost NEFA were standardized to the membrane protein measured by the Lowry technique [28], with bovine serum albumin (Sigma) as a standard. All solvents used in the lipid extractions, preparative chromatography, and HPLC were HPLC grade and obtained from Fisher Scientific (Medford, Massachusetts, USA).

Red cell cation flux measurements

RBCs suspended in the preservation buffer were used in a cation flux assay within 24 hours of venipuncture. The RBC electrolyte content was always determined within two hours of venipuncture. ^{86}Rb influx into, or Na efflux from, erythrocytes was measured using either fresh cells RBCs or RBCs loaded with Na^+ to measure the V_{max} of the Na^+ pump (loaded cells).

Sodium efflux. Sodium efflux into choline-containing media and intracellular electrolyte content were measured as previously described [29, 30]. For assays at the V_{max} of the Na^+ pump, RBCs were loaded to contain 50 mmol/liter cell of Na^+, as previously described [31]. The nystatin loading solution contained 70 mM KCl, 70 mM NaCl and 50 mM sucrose. Ouabain-sensitive Na^+ flux was measured by incubating the cells in 10 mM KCl in choline media [31]. For measurement of lithium-sodium countertransport, RBCs were loaded with 10 mM LiCl and 130 mM KCl in the presence of nystatin, and the efflux of lithium into a choline and Na^+ medium was measured, as previously described [29]. Following Li^+ loading, the intracellular Li^+ concentration was 6.7 ± 0.5 mmol/liter pre-dialysis and 6.9 ± 0.4 mmol/liter post-dialysis. Nystatin was removed by incubating loaded cells in a washing solution containing 0.1 g/100 ml albumin (bovine, fraction V, Sigma) of similar ionic composition as the loading solution. $Na^+/K^+/Cl^-$ cotransport was determined by furosemide-sensitive Na^+ efflux into choline media containing ouabain, as previously described [30].

In 20 patients from Group 1, ouabain-sensitive Na^+ efflux rates were also determined in aliquots of pre-dialysis red cells that had been pre-incubated in delipidated albumin. Following Na^+ loading of cells and removal of the nystatin using Cohn fraction V, non-delipidated, bovine serum albumin, an aliquot of cells was resuspended in buffer containing 70 mM NaCl, 70 mM KCl, 10 mM Tris-Mops (pH 7.4 at 37°C), 50 mM sucrose, 0.1 mM KPO_4, 10 mM glucose, 1.0 mg/ml fatty acid free bovine serum albumin (Sigma #A7030), and incubated at 37°C for 30 minutes. These cells were then washed with CWS five times

and used in parallel determinations of cation flux rates, [^{3}H]ouabain binding, and RBC membrane NEFA content.

^{86}Rb influx. Because the albumin used to remove the ionophore, nystatin, from RBC membranes after loading with Na^+ could influence cation flux rate by removing NEFA from the membrane (**Results**), a different procedure was used. Because treatment with 2,5-p-chloro-mercuribenzene sulfonate (PCMBS) has been used extensively to study Na^+ pump kinetics [32], we used this procedure in parallel incubations in cells obtained from 10 hemodialysis patients. Rb influx into fresh RBCs was determined by incubating red cells in 150 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.4 at 37°C), 10 mM glucose, and 4 mM KCl with 100 μCi ^{86}Rb, with and without 0.1 mM ouabain or 0.1 mM ouabain and 1 mM furosemide. Triplicate samples were obtained after five minute and 25 minute incubations at 37°C in a shaking water bath. The RBCs were washed three times with cold influx media, and then the cell pellet was lysed with 0.02% Acationox and centrifuged. Aliquots of the cell lysate were used for counting ^{86}Rb and optical density of hemoglobin for determination of the red cell volume. ^{86}Rb was determined by gamma counting. The specific activity of ^{86}Rb/K^+ was used to determine the influx in mmol/liter cell/hr.

Red cell [^{3}H]ouabain binding

Aliquots of packed RBCs were resuspended in a buffer containing 150 mM NaCl, 1 mM $MgCl_2$, and 10 mM Na^+ phosphate (pH 7.4 at 37°C) and washed twice. RBCs (0.5 ml) resuspended in this buffer (25% hematocrit) were added to tubes containing [^{3}H]ouabain (20 Ci/mmol, New England Nuclear, Bedford, Massachusetts, USA; 10^{-7} M final concentration) in 0.5 ml of buffer with or without 0.1 mM unlabelled ouabain and incubated for one hour at 37°C. RBC number was determined using a Coulter Counter (Coulter Electronics, Hialeigh, Florida, USA). Following the incubation, the cells were washed twice in 2 ml of iced Na^+ phosphate buffer solution (pH 7.4 at 4°C), and 0.2 ml of 10% perchloric acid were added to extract bound [^{3}H]ouabain. Radioactivity was determined by scintillation counting with correction for quenching using an external standard.

Patients

Subjects participating in this study were stable hemodialysis patients who were not receiving digitalis and had not received a blood transfusion within two weeks of the study. All patients were receiving three dialysis treatments per week. Approximately 60% were dialyzed with a bicarbonate containing bath (in mmol/liter: Na 135, Ca 3, Mg 0.75, Cl 104.8, bicarbonate 35) and 40% with an acetate containing bath (in mmol/liter: Na 136.5, Ca 3, Mg 0.75, Cl 103.1, acetate 39.2). The concentrations of dextrose and K^+ were varied according to each patient's clinical condition and plasma K^+ level. Erythrocytes from 28 age-matched ambulatory patients with normal renal function were also studied to determine [^{3}H]ouabain binding and intracellular Na^+ content. This study was approved by the Human Subjects Committee of the Brigham and Women's Hospital.

Statistics

Data are presented as the mean ± SEM and statistical evaluation was based on paired observations using the Student's *t*-test, unless otherwise noted; the null hypothesis was rejected if $P < 0.05$.

Table 1. Clinical characteristics of the dialysis patients

	Group 1 *Na^+-loaded cells*	Group 2 *fresh cells*
Number of patients	34	17
Age *yr*	65.7 ± 3.7	61.1 ± 4.9
Sex	20 W/14 M	11 W/6 M
Weight change with dialysis *kg*	1.6 ± 0.18	1.6 ± 0.35
Serum Na^+ *mmol/liter*	135.0 ± 0.6	133.4 ± 1.1
Serum K^+ *mmol/liter*	5.1 ± 0.2	4.9 ± 0.3
Serum Cl *mmol/liter*	99.8 ± 1.0	98.7 ± 1.6
Serum HCO_3 *mmol/liter*	20.0 ± 0.6	20.7 ± 1.0
BUN *mg/dl*	72.4 ± 4.0	84.6 ± 9.8
Serum Cr *mg/dl*	8.7 ± 0.4	10.0 ± 1.1
Albumin *g/dl*	3.3 ± 0.1	3.2 ± 0.1

Values are mean ± SEM.

Table 2. The effects of hemodialysis on intracellular Na^+ and K^+ content of fresh cells

	Pre-dialysis	Post-dialysis	*P*
Group 1			
Na^+	9.2 ± 0.5	9.4 ± 0.5	NS
K^+	101.9 ± 1.7	102.6 ± 1.7	NS
Group 2			
Na^+	9.4 ± 0.7	9.9 ± 0.9	NS
K^+	99.4 ± 1.8	100.8 ± 2.3	NS

Values are mean ± SEM in mmol/liter cell prior to Na^+ loading with nystatin and/or PCMBS (**Methods**) for 34 patients in Group 1 and 17 patients in Group 2.

Results

The clinical characteristics of the 51 patients studied are shown in Table 1. Maximal rates of Na^+ transport in Na^+-loaded erythrocytes, as well as plasma and cell membrane NEFA content were studied in 34 hemodialysis patients (Group 1, Table 1). In a second group of 14 hemodialysis patients (Group 2, Table 1), ^{86}Rb influx into fresh cells was studied to determine whether acute changes in intracellular Na^+ content could account for some of the changes in active Na^+ transport that occur with dialysis. Aside from the higher percentage of women in Group 2, the two study populations were not different in other clinical parameters. Fourteen patients in Group 1 were Black and four were Hispanics, compared to eight and two in Group 2, respectively. There was no apparent correlation between race and any cation flux parameter. There was also no correlation between the composition of the dialysis bath (acetate or bicarbonate) and changes in either plasma membrane NEFA levels or cation flux rates.

Effect of dialysis on cation transport

There was no significant change in intracellular Na^+ or K^+ with hemodialysis in Group 1 patients or Group 2 patients (Table 2). In 10 of 34 Group 1 patients, erythrocyte cation transport pre- and post-dialysis were assessed by measuring ^{86}Rb influx, as well as Na^+ efflux. In these patients, the two methods commonly used for Na^+ loading, PCMBS and nysta-

Table 3. Changes in red cell cation transport and in [^{3}H]ouabain binding during hemodialysis

	Pre-dialysis	Post-dialysis	% Change	*P*
Group 1				
Maximal rates				
Na^+ pump activity[a]	4.10 ± 0.24	4.31 ± 0.27	6.9 ± 5.9	NS
Na^+/K^+ cotransport[b]	0.56 ± 0.06	0.69 ± 0.07	25.0 ± 9.9	<0.02
Na^+/Li^+ countertransport[c]	0.39 ± 0.04	0.41 ± 0.04	12.3 ± 10.0	NS
[^{3}H]ouabain binding	320 ± 36	321 ± 21	−3.1 ± 8.4	NS
Group 2				
Fresh cells				
Na^+ pump activity[a]	1.72 ± 0.12	1.68 ± 0.14	−4.5 ± 6.4	NS
[^{3}H]ouabain binding	328 ± 19	316 ± 20	−2.2 ± 4.7	NS

Values are mean ± SEM from 34 patients in Group 1 (Na^+/Li^+ countertransport was studied in 12) and 17 patients in Group 2. All ion fluxes are expressed in mmol/liter cell per hr and ouabain-binding as sites per cell.

[a] Ouabain-sensitive Na^+ efflux (Group 1) or ouabain-sensitive ^{86}Rb influx (Group 2)

[b] Furosemide-sensitive Na^+ efflux

[c] Li efflux stimulated by external Na^+. In Group 2, Na^+ pump activity was determined as ouabain-sensitive ^{86}Rb influx into fresh cells.

tin, were compared to assess whether the nystatin method, in which the ionophore is removed with albumin, could alter red cell membrane NEFA content. In nystatin-loaded erythrocytes, intracellular Na^+ and K^+ were 37.4 ± 1.1 and 40.2 ± 4.2 mmol/liter, respectively. In cells loaded using PCMBS, the final Na^+ and K^+ concentrations were slightly, but significantly, higher (46.0 ± 1.7 mmol/liter, Na^+, and 49.3 ± 2.2 mmol/liter, K^+; $P < 0.01$). Because pump-mediated Rb influx saturates over 30 mmol/liter cell of Na^+, these differences should not influence ouabain-sensitive cation flux rates. In fact, ouabain-sensitive ^{86}Rb influx in cells loaded with either technique (nystatin: 2.6 ± 0.23 vs. PCMBS: 2.9 ± 0.20 PCMBS mmol/liter cells/hr) were not different. Similarly, there was no difference in furosemide-sensitive ^{86}Rb influx using either method (0.23 ± 0.02 nystatin vs. 0.21 ± 0.02 PCMBS mmol/liter cells/hr). As the two techniques yielded similar results, the remainder of the cation flux experiments in Group 1 patients were performed with the technically more convenient nystatin method.

In Na^+-loaded RBCs, maximal Na^+ pump activity (Group 1 patients) did not change significantly with hemodialysis (6.9 ± 5.9% increase, Table 3). Similarly, in fresh cells from Group 2 patients, there was no significant change in ouabain-sensitive Na^+ efflux rates following dialysis (−4.5 ± 6.4% change, Table 3). However, in Group 2 patients, there was a significant correlation with changes in intracellular Na^+ occurring with dialysis and ouabain-sensitive Na^+ efflux in fresh, nonNa^+-loaded erythrocytes ($y = 0.71x - 2.4$; $r = 0.59$; $P < 0.01$, $N = 17$).

The effect of dialysis on the activity of $Na^+/K^+/Cl^-$ cotransport was examined in all Group 1 patients. There was a small, but significant, rise in the furosemide-sensitive influx (0.14 ± 0.06 mmol/liter cell/hr, $P < 0.02$; Table 3). There was no apparent relationship between intracellular electrolytes (before or after loading) and $Na^+/K^+/Cl^-$ cotransport maximal activity in loaded cells, nor was there any correlation between plasma K^+ and furosemide-sensitive Na^+ efflux in these cells. Measurement of sodium-lithium countertransport in 12 of 34 Group 1 patients showed no changes during hemodialysis (Table 3).

Twenty patients in Group 1 were studied on several occasions over two years. Although in 11 of these subjects, predialysis ouabain-sensitive Na^+ efflux rates improved (+1.15 ± 0.47 mmol/liter cell/hr), there was no significant improvement in Na^+ pump activity in the group as a whole. There also was no significant change in $Na^+/K^+/Cl^-$ cotransport over this time.

Effect of dialysis on [^{3}H]ouabain binding

The number of specific [^{3}H]ouabain binding sites was determined in both Group 1 and Group 2 patients. No significant change in the number of [^{3}H]ouabain binding sites was induced by hemodialysis in either group (Table 3), and there was no correlation between the number of [^{3}H]ouabain binding sites and the intracellular Na^+ concentration. As expected, there was a good correlation between maximal ouabain-sensitive Na^+ efflux in Na^+-loaded cells and the number of [^{3}H]ouabain binding sites in erythrocytes from Group 1 patients ($r = 0.754$, $P < 0.001$). When data from all Group 1 and 2 patients were compared to those from 28 ambulatory patients with normal renal function, there were no differences in either the intracellular Na^+ concentration or the number of [^{3}H]ouabain binding sites.

Changes in plasma NEFA with dialysis

Plasma concentrations of NEFA were within the normal range before dialysis (0.57 ± 0.05 mM), and largely consisted of palmitic, stearic, and oleic acids, with an unsaturated-to-saturated ratio of 0.95 ± 0.09 (Table 4). Post-dialysis and following the administration of an average dose of 3990 ± 344 units of heparin, the mean plasma NEFA concentration nearly doubled (+87%), increasing in 29 of 34 patients (Table 4, Fig. 1A). The ratio of unsaturated-to-saturated NEFA in plasma also significantly increased, rising 54% to 1.5 ± 0.09; the largest increases were due to oleic and linoleic acids. There was no correlation between the plasma levels of NEFA measured either before or just after dialysis and cation flux rates. There was also no relationship between the magnitude of the change in plasma NEFA with dialysis and any change in RBC cation transport.

Changes in erythrocyte membrane NEFA with dialysis

Erythrocyte membrane NEFA were easily detectable using the fluorescent derivatizing compound, Br-Mac, and ranged from 10 to 100 nmol/mg protein. The predominant red cell membrane NEFA were palmitic and stearic, with a much lower ratio of unsaturated-to-saturated NEFA than that present in plasma (Table 4).

In contrast to the rise in plasma NEFA concentration with dialysis, the concentration of NEFA in RBC membranes fell in 28 of 34 patients; the average decrease was 23% (Table 4, Fig. 1B, $P < 0.001$). Although the magnitude of the rise in plasma NEFA did not correlate with the change in RBC membrane NEFA, all but 8 of the 34 patients with a decline in RBC membrane NEFA experienced an increase in plasma NEFA. There was no change in the ratio of unsaturated-to-saturated NEFA in erythrocyte membranes with dialysis (Table 4), nor was there any correlation between changes in membrane and plasma unsaturated-to-saturated NEFA ratios.

There was no apparent relationship between total erythrocyte

Table 4. Plasma and red cell membrane NEFA content changes during hemodialysis

	Pre-dialysis	Post-dialysis	% Change	*P*
Plasma				
Total NEFA content *μmol/liter*	569.9 ± 52.2	1063.9 ± 120.1	86.8 ± 13.2	<0.001
Unsaturated/saturated NEFA ratio	0.946 ± 0.090	1.46 ± 0.088	54.3 ± 2.1	<0.001
Erythrocyte membrane				
Total NEFA content *nmol/mg protein*	71.7 ± 8.1	56.1 ± 7.0	−22.8 ± 3.8	<0.001
Unsaturated/saturated NEFA ratio	0.383 ± 0.045	0.398 ± 0.038	3.9 ± 0.3	NS

Values are mean ± SEM for 34 patients in Group 1. Statistical differences were analyzed by paired Student's *t*-test.

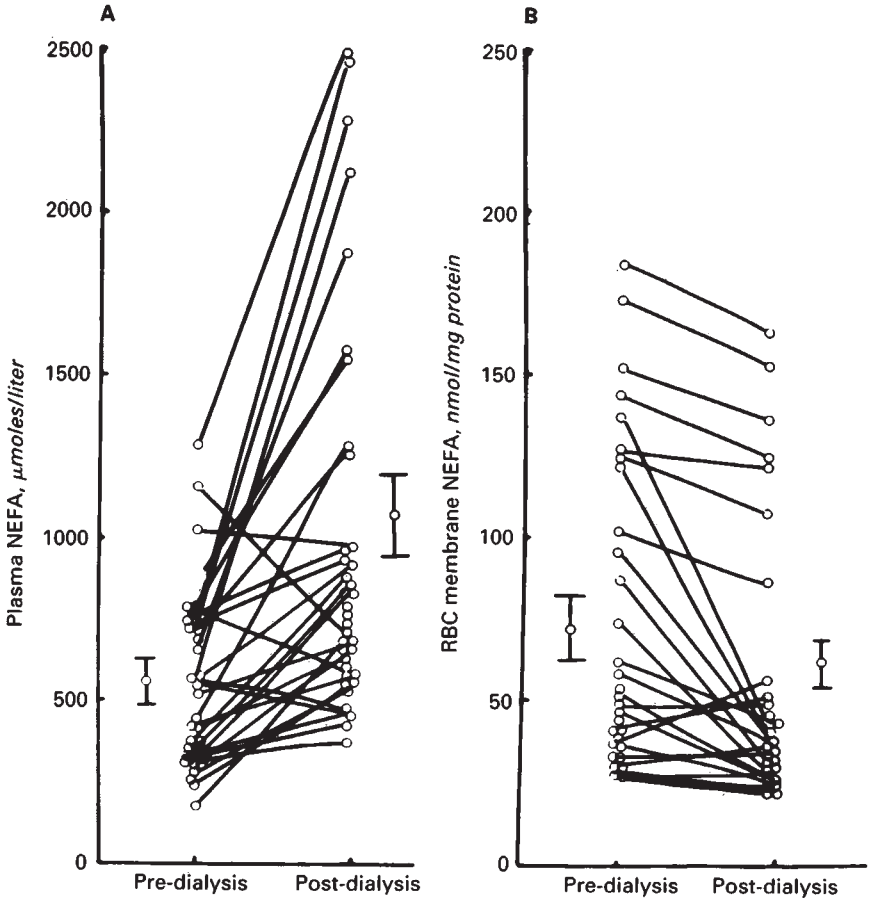

Fig. 1. *The rise in plasma NEFA with dialysis in 34 patients in Group 1 is illustrated in* **(A)**. The percent change averaged 86.8 ± 13.2% ($P < 0.001$). In contrast, in **(B)**, RBC membrane NEFA fell in the same patients by an average of 23% with hemodialysis ($P < 0.001$, by paired Student's *t*-test).

membrane NEFA levels and ouabain-sensitive efflux rates pre- or post-dialysis. However, when the percent change in erythrocyte membrane NEFA was plotted against the percent change in Na^+ pump activity, a possible relationship could be discerned (Fig. 2A). Nine of 11 patients with greater than a 30% decrease in membrane NEFA had an increase in ouabain-sensitive efflux, while four of five patients with an increase in red cell membrane NEFA during dialysis had a decline in Na^+ pump activity. Indeed, when the percent change in Na^+ activity was plotted against the log of the percent decline in red cell NEFA in the 24 of the 34 subjects who had at least a 10% decrease in membrane NEFA, there was a significant, positive correlation ($r = 0.56$, $N = 24$, $P < 0.02$; Fig. 2B). There was no significant correlation between changes in red cell membrane NEFA and changes in either furosemide-sensitive Na^+ efflux rates, nor in changes in Na^+/Li^+ countertransport.

In 20 patients randomly chosen from the 34 patients in Group 1, erythrocytes obtained before a dialysis treatment were incubated in fatty acid free albumin, and changes in ouabain-sensitive cation flux and red cell membrane NEFA levels were compared to the effects of hemodialysis itself on these same parameters. Incubation with delipidated albumin resulted in a 15.9 ± 3.5% fall in red cell membrane NEFA content, a value closely correlated to the decrease in membrane NEFA induced by dialysis on the same day in the same patients ($r = 0.84$, $P < 0.001$; Table 5). Although there was no linear relationship

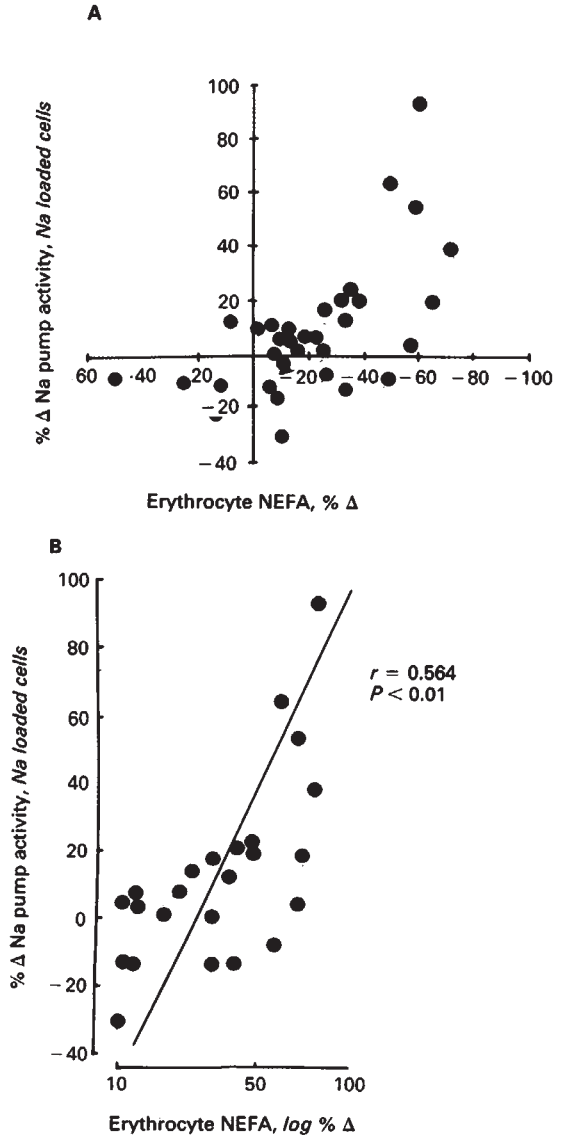

Fig. 2. *In* **(A),** *the percent change in Na^+ pump activity (ouabain-sensitive Na^+ efflux) is plotted against the percent change in RBC membrane NEFA.* Although there is no significant change in average Na^+ pump activity with dialysis (Table 3) nor any linear correlation between the two variables shown in (A), there is a significant correlation when the log of the percent decline in erythrocyte NEFA is plotted against the percent change in Na^+ pump activity, as shown in **(B)** (r = 0.564; $P < 0.01$) in 24 of the 34 subjects studied who had a decrease in membrane NEFA content >10%.

between decreases in RBC membrane NEFA content and changes in sodium pump flux with dialysis, a significant correlation between these two parameters was detected in predialysis, erythrocytes incubated in albumin (r = 0.54, $P < 0.05$; Table 5). Interestingly, the changes in ouabain-sensitive cation flux induced by incubation in delipidated albumin statistically were related to the changes in ouabain-sensitive cation transport that occurred with dialysis in the same patients (r = 0.47, $P < 0.05$; Table 5).

Table 5. Changes in red cell membrane NEFA content and NA pump activity with incubation in delipidated albumin and during hemodialysis

	RBC membrane NEFA (*nmol/mg*)	RBC Na^+ pump *mmol/liter cell/hr*	Correlation % *ΔNEFA:% Δ pump*
Pre-dialysis	68.2 ± 7.7	4.08 ± 0.26	—
Post-dialysis	59.1 ± 7.2	4.37 ± 0.29	
% Change	13.5 ± 2.8%	4.2 ± 6.0%	r=0.41
P	< 0.01	NS	NS
Post-albumin	57.4 ± 9.1	4.35 ± 0.31	
% Change	15.9 ± 3.5%	6.7 ± 5.9%	r=0.54
P	< 0.01	NS	< 0.05
Correlation %Δ dialysis:% Δalbumin P	r = 0.84 < 0.01	r = 0.47 < 0.05	—

Values are mean ± SEM for 20 patients in Group 1. RBC sodium pump activity is ouabain-sensitive sodium efflux from sodium-loaded cells.

Discussion

We have studied the acute effects of a hemodialysis procedure on erythrocyte cation flux rates in stable hemodialysis patients. In agreement with several other reports [3, 4, 7], erythrocyte Na^+ and K^+ contents were not increased on average in these patients, nor was there any correlation between the intracellular Na^+ content and either the number of [^{3}H]ouabain binding sites or the maximal ouabain-sensitive Na^+ efflux rate. Since there was a limited number of patients studied with a very high erythrocyte Na^+ content, we cannot exclude the possibility, as suggested by Cheng, Kahn and Kaji [3], that there is a subset of uremic dialysis patients with high cell Na^+ and significantly depressed Na^+ pump number and activity.

Compared to rates of ouabain-sensitive Na^+ efflux in erythrocytes from normotensive subjects with normal renal function as previously reported by us (5.10 ± 0.24 mmol/liter cells/hr, ouabain-sensitive Na^+ efflux; N = 21) [29, 30], average active Na^+ efflux rates were moderately decreased in RBCs from patients in the current study. However, Na^+ pump activity did not change acutely with hemodialysis whether assayed as changed in ouabain-sensitive cation flux in fresh cells (Table 3, Group 2) or at the V_{max} of the Na^+ pump in Na^+-loaded cells (Table 3, Group 1). Although others have reported a correlation between weight loss with dialysis and change in active Na^+ transport [4], we could not confirm this in either group of patients. There was no relationship as well between changes in extracellular volume status with dialysis as reflected by weight loss and changes in $Na^+/K^+/Cl^-$ cotransport activity. However, as expected, there was a significant correlation between changes in the intracellular Na^+ content and Na^+ pump activity in fresh cells, indicating that either increases or decreases in intracellular Na^+ during the dialysis procedure can be of sufficient magnitude to influence ouabain-sensitive Na^+ efflux acutely in fresh cells.

Furosemide-sensitive $Na^+/K^+/Cl^-$ cotransport rates were much lower in the patients we studied than in normal subjects we have previously reported (normal values for furosemide-sensitive Na^+ efflux rates of 0.978 ± 0.093 mmol/liter cells/hr, N = 21) [29, 30], in agreement with evidence from several other groups [7, 13, 33]. In contrast to the Na^+ pump data, there was a small, but significant, increase in furosemide-sensitive cotransport rates that occurred acutely with hemodialysis (Table 3,

Group 1). As these measurements were performed in pre- and post-dialysis RBCs loaded to contain the same intracellular Na^+ and K^+, and since the osmolality of all solutions was kept constant, changes in plasma K^+ or red cell volume with dialysis were unlikely to be the cause of the increase in furosemide-sensitive Na^+ efflux rates. Finally, in contrast to the reports of Woods, Parker and Watson [5] and Trevisan et al [6], there were no changes in maximal red cell Na^+/Li^+ countertransport rates with dialysis. This concurs with recent reports by Corry et al [7] and Smith et al [9].

The goal of the present study was to determine whether acute changes in erythrocyte cation flux rates in end-stage renal failure patients undergoing a hemodialysis procedure could be accounted for by acute changes in red cell membrane NEFA content. NEFA, particularly those with at least one cis-unsaturated double bond, are known to affect NaK-ATPase enzyme activity in broken membrane preparations [21, 34]. The effect is most likely due to NEFA interactions with the lipid bilayer that result either in changes in the viscosity of the bilayer as a whole, or in specific lipid domains adjacent to the enzyme that are sufficient to alter enzyme conformation. NEFA can also induce conformational changes in the enzyme sufficient to inhibit cardiac glycoside binding [20].

The content of NEFA and other polar lipids, such as lysophospholipids, normally does not exceed 1 to 2 mol percent of the total phospholipid in the erythrocyte membrane [35]. In nucleated cells, NEFA that partition into the cell membrane or arise from the activity of membrane phospholipases are rapidly converted to acyl CoA derivatives for re-esterification into phospholipids or triglyceride, or combined with carnitine for eventual beta-oxidation in mitochondria. In contrast, the red cell does not have as many metabolic options and may be less able to respond to rapid changes in plasma NEFA levels or, perhaps more importantly, to changes in the binding of NEFA to plasma proteins, principally albumin.

Not surprisingly, total plasma NEFA levels rose significantly following hemodialysis, in part because of the heparin administered combined with the abnormal clearance of triglycerides characteristic of these patients [22, 36]. Artifactually high NEFA levels have been reported to occur in plasma samples from patients who have received systemic heparinization due to continuing activity of activated lipoprotein lipase in vitro following phlebotomy. Although we cannot exclude an effect of activated lipoprotein lipase on NEFA production in our assay samples, care was taken to draw venous blood into pre-chilled tubes, all transfers of plasma were kept on ice, and polar lipids were extracted in less than one hour following phlebotomy.

Another explanation for the rapid rise in total plasma NEFA levels during dialysis is that the number of albumin binding sites available to bind NEFA increased. Albumin, the primary carrier of NEFA in plasma, contains a heterogenous assortment of both high-affinity and low-affinity NEFA binding sites [37]. Albumin normally carries 0.5 to 2.0 mol NEFA per mol of protein, although much higher mol ratios of NEFA to albumin have been obtained in vitro [37]. NEFA transfer between artificial lipid bilayers and albumin occurs rapidly, particularly if NEFA are in the ionized form, as would occur at physiologic pH. Occupation of organic acid binding sites on albumin by accumulated metabolic by-products could result in displacement of NEFA from at least low affinity sites. Removal of organic acids by dialysis could cause transfer of NEFA from cell membranes to albumin and result in a rise in total plasma NEFA levels and a fall in RBC membrane NEFA levels, despite a heparin-induced increase in lipoprotein lipase activity. This is, in fact, what we found: RBC membrane NEFA decreased by an average of 23%, a decline that was highly significant by paired *t*-test. In addition, the fall in red cell NEFA content occurring with dialysis could be mimicked by incubating RBCs obtained before dialysis with delipidated albumin (Table 5). There was also a significant correlation between changes in Na^+ pump activity occurring with hemodialysis, and changes in Na^+ pump activity occurring during incubation of the same pre-dialysis erythrocytes with fatty acid-free albumin. These data support the contention that increased plasma protein binding of NEFA following dialysis removes NEFA from cell membranes and contributes to the rise in total plasma NEFA levels.

Although there was no significant change in Na^+ pump-mediated Na^+ efflux with dialysis in this patient population as a whole, 24 of the 34 subjects studied had a decrease in erythrocyte membrane NEFA content of greater than 10% and in these patients, there was a significant correlation between the log of the percent decline in membrane NEFA and the percent change in maximal Na^+ pump activity (Fig. 2B). Smaller decreases or increases in membrane NEFA did not have a discernible effect on ouabain-sensitive Na^+ efflux. These data suggest that if variations in membrane NEFA content are to have any effect on the function of membrane proteins, the change must be relatively great.

There are several possible reasons as to why the correlation between changes in membrane NEFA levels and Na^+ pump activity is better than that observed here. The first is that there may be a threshold effect; that is, a relatively large shift of NEFA out of the membrane (or out of specific membrane domains) is necessary before any effect on membrane transport proteins can be detected. Secondly, if lipid-protein interactions are important in affecting membrane transport proteins, a relatively high cholesterol:phospholipid ratio in erythrocytes from some uremic patients may mask or dampen the effect of alterations in the polar lipid content of membranes [34, 38, 39]. Indeed, cholesterol has been demonstrated to have an independent effect on Na^+ pump activity in red cell membranes [40]. Similarly, a reduction in the phospholipid to sphingomyelin ratio, as has been reported to occur in uremic erythrocytes [41], will also tend to increase the microviscosity of the membrane. This would minimize the effect of acute changes in NEFA content in some patients, and possibly independently modulate NaK-ATPase activity, as has been reported in cultured myocardial cells [42]. Finally, changes in membrane NEFA content may not be causally related to changes in NaK-ATPase activity, but may only be a marker for changes in other polar lipids, such as lysophospholipids, that are also known to affect membrane protein function. Nevertheless, the recent demonstration that L-carnitine dietary supplements can improve ouabain-sensitive sodium efflux rates in erythrocytes from hemodialysis patients supports our contention that altered NEFA metabolism plays a role in abnormal cation flux in uremia [43].

In summary, the evidence presented here supports the possibility that NEFA, and perhaps other polar lipids, affect active cation transport rates in uremic erythrocytes. There are very likely other reasons as well for altered cation flux rates in renal

failure. Further studies are needed to clarify the role of abnormal membrane lipid-protein interactions in the pathophysiology of uremia.

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